#22

Our Ref.: 427.010-1-Div-2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: :
BIGG et al : Examiner: S. Dodson
Serial No.: 09/612,382 :
Filed: July 7, 2000 : Group: 1624
For: NEW ... CONTAINING THEM :
600 Third Avenue
New York, NY 10016

DECLARATION

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Christophe Thurieau hereby deposes and says as follows:

That he has been employed by Beaufour Ipsen Group since October 1996 and is the Director of Research of the said company.

That the following tests were conducted under his supervision and he has reviewed the following data which are deemed to accurately reflect the anti proliferative activity of over 130 different homo-camptothecin analogs.

TEST DATA

The anti-cancer activity of homo-camptothecins i.e. camptothecin analogs having a 7-ring member ß-hydroxy lactone ring of the formula according to the present invention have been estimated by their *in vivo* cell proliferation on various cell lines : the IC50 value has been measured according to the classical colorimetric test in this field (Tetrazolium salt :MTT or WST1)(J Immunol Methods 1983 Dec 16 ; 65(1-2) : 55-63 - Cancer Res 1988 Sep 1;48(17) : 4827-33 - Nippon Ronen Igakkai Zasshi 1998

Jul;35(7) :535-42 (abstract in English in Medline database under N° 1998451976)).

- for bladder, breast, CNS, colon, leukemia, lung and prostate, 3 tests have been conducted on one or different cell lines : the mean of IC50 thus obtained is expressed as a specific number in nM (nanomolar) or as a margin [x-y], the IC50 being comprised between 10^{-y}M and 10^{-x}M.

- for stomach, melanoma and ovarian, 2 tests have been conducted on one cell line : the results of each test and the mean of IC50 thus obtained are expressed in nM.

Examples 1-187 belong to co-pending US patent application 10/071,046. Examples 1-82 belong to US patent application 09/612,382. Examples A to F are additionnal examples falling within the scope of US patent application 09/612,382 but not specifically disclosed therein (see enclosed list).

CONCLUSION

The results as set forth in the tables attached hereto and incorporated herein by reference indicate that the 137 tested compounds exhibit a measurable anti-proliferative activity against most of the ten different malignant strains that were tested.

These results confirm that the homo-camptothecins having a 7-ring member ß-hydroxy lactone ring of the formula according to the present invention are useful anti-cancer products.

It is declared by the undersigned that all statements made herein of undersigned's own knowledge are true and that all statements made on information and belief are believed to be true;

and further that these statements made were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S. Code § 1001, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Dated: This 3rd day of May, 2002.

Christophe Thurieau

Christophe Thurieau

CAM:sd

List of additionnal examples :

Example A : 5-ethyl-5,10-dihydroxy-11-[(1-pipéridinyl)methyl]-4,5,13,15-tetrahydro-1*H*,3*H*-oxepino [3',4':6,7] indolizino [1,2-*b*] quinoleine-3,15-dione

Example B : 5-ethyl-9-chloro-10-fluoro-5-hydroxy-12-[(1-pipéridinyl)methyl]-4,5,13,15-tetrahydro-1*H*,3*H*-oxepino [3',4':6,7] indolizino [1,2-*b*] quinoleine-3,15-dione

Example C : 5-ethyl-5,10-dihydroxy-11-[(4-methyl-1-piperazinyl)methyl]-4,5,13,15-tetrahydro-1*H*,3*H*-oxepino [3',4':6,7] indolizino [1,2-*b*] quinoleine-3,15-dione

Example D : 5-ethyl-10-methoxy-5-hydroxy-12-[(1-pipéridinyl)methyl]-4,5,13,15-tetrahydro-1*H*,3*H*-oxepino [3',4':6,7] indolizino [1,2-*b*] quinoleine-3,15-dione

Example E : (5*R*)-5-ethyl-10-chloro-5-hydroxy-12-(2-chlorophenyl)-4,5,13,15-tetrahydro-1*H*,3*H*-oxepino [3',4':6,7] indolizino [1,2-*b*] quinoleine-3,15-dione

Example F : (5*R*)-5-ethyl-9,11-difluoro-5-hydroxy-12-phenyl-4,5,13,15-tetrahydro-1*H*,3*H*-oxepino [3',4':6,7] indolizino [1,2-*b*] quinoleine-3,15-dione

	Bladder		Breast			CNS	Colon	Leukemia	Lung		Prostate	
Example	T24s	T24r	MCF7s	MCF7ras	MCF7 mdr	SK-N-SH neuroblastoma	HT29	K562 /Adr	A427	Calu-1	PC3	DU145
3			*[6-7]*		*[6-7]*		*[8-9]*	*[8-9]*	*[8-9]*	*[6-7]*	*[7-8]*	*[8-9]*
9			*[5-7]*		*[5-7]*	*[9-11]*	[6-7]	*[8-9]*	*[9-10]*	*[6-7]*	*[6-8]*	[6-8]
11							[6-7]					
12			[6-7]				[6-8]	[6-7]	[7-9]		[6-7]	[8-9]
15			*[4-5]*		*[4-5]*		*[7/8]*	*[5-6]*	*[7-8]*	*[4-6]*	*[5-6]*	*[7-8]*
32 (HCl salt)			[5-6]	[5-6]	[5-6]	[5-6]	[6-7]	[<5]	[7-8]	[<5]	[6-7]	[7-8]
32			[5-6]	[5-6]	[5-6]	[5-6]	[6-7]	[5-6]	[7-8]	[<5]	[5-6]	[7-8]
23			[7-8]	[7-8]	[9]	[7-8]	[8-9]	[8-9]	[11-13]	[7-8]	[13?]	[10-11]
14			[5-6]	[5-6]	[6-7]	[5-6]	[5-6]	[5-6]	[7-8]	[<5]	[6-7]	[7-8]
20			[6-8]	[6-8]	[7-9]	[7-8]	[8-9]	[6-8]	[11-12]	[6-7]	[13?]	[10-11]
21			[6-7]	[6-7]	[7-8]	[6-7]	[7-8]	[6-7]	[8-11]	[6-7]	[8-9]	[8-9]
37			[5-7]	[6-7]	[5-6]	[6-7]	[7-8]	[<5]	[8-10]	[5-6]	[7-8]	[8-9]
34			[5-7]	[5-7]	[5-6]	[6-7]	[6-8]	[5-6]	[8-9]	[5-6]	[9-10]	[8-9]
16			[7-8]	[7-8]	[8-9]	[7-8]	[8-9]	[6-8]	[10-12]	[7-8]	[13?]	[10]
24			[6-7]	[6-7]	[7-8]	[6-7]	[7]	[6-7]	[8-9]	[6-7]	[10-11]	[8-9]
29			[6-8]	[6-8]	[7-9]	[6-7]	[8-9]	[7-8]	[9-11]	[6-7]	[11-12]	[9-10]
18	**18,80**	**4,87**	**[6-7]**	**[6-7]**	**[8-9]**	**[6-7]**	**[7-9]**	**[7-8]**	**58,90**	**[6-7]**	**32,30**	**6,10**
18			**29,10**		**713,00**		**26,60**	**390,00**	**24,50**		**112,00**	**4,27**
26			[6-8]		[8-11]	[6-7]	[9-11]	[8-9]	[9-12]	[5-7]	[8-9]	[9-11]
A			[5-6]		[5-6]	[5-6]	[6-7]	[5-6]	[7-8]	[<5]	[6-7]	[7-8]
25			[6-7]		[8-10]	[6-7]	[8-10]	[5-6]	[9-10]	[5-7]	[8]	[9-10]
17			[7-8]		[10-13]	[6-7]	[8-10]	[8-9]	[10-13]	[6-7]	[8-9]	[10-13]
40			[5-7]		[5-6]	[5-7]	[5-7]	[<5]	[8-9]	[<5]	[6-7]	[7-9]
33			[6-7]		[6-7]	[5-6]	[6-8]	[5-7]	[7-8]	[5-6]	[6-7]	[8-9]
42			[6-7]		[8-9]	[6-7]	[7-9]	[7-9]	[8-9]	[5-7]	[7-8]	[8-10]
3 ((5S) enantiomer)			[5-6]		[6-7]	[5-6]	[5-6]	[5-7]	[6-7]	[<5]	[5-6]	[6-7]
38			[5-7]		[5-6]	[6-7]	[6-8]	[6-7]	[7-9]	[<5]	[8]	[8-9]
76			[<5]		[5-6]	[5-6]	[5-6]	[5-6]	[7-8]	[<5]	[5-7]	[7-8]
22			[6-7]		[8-9]	[6-7] ; [10-11]	[7-8]	[7-8] ; [11-12]	[8-9]	[6-7]	[7-8] ; [12-13]	[8-9]
27			[<5]		[<5]	[<5]	[5-6]	[<5]	[6-7]	[<5]	[5-6]	[6-7]
19			[7-8]		[9-10]	[7-8]	[7-9]	[7-9]	[9-10]	[6-8]	[8-9] ; [13]	[9-11]
30			[5-7]		[5-6]	[5-6]	[6-7]	[4-6]	[7-8]	[<5]	[6-9]	[8-10]
31			[6-7]		[6-7]	[6-7]	[6-9]	[5-7]	[8]	[5-6]	[7-8] ; [11-13]	[7-10]
35			[5-6]		[<5]	[5-6]	[5-8]	[5-7]	[6-8]	[<5]	[6-7]	[7-8]
28			[7-8]		[8-9]	[6-7]	[8-11]	[7-9]	[9-11]	[6-8]	[8-9]	[9-11]
65			[6-7]		[6-7]	[5-8]	[7-9]	[5-8]	[7-9]	[5-6]	[7-8]	[9-11]
174			[6-7]		[8-9]	[6-8]	[8-9]	[6-8]	[7-9]	[5-7]	[7-8]	[9-10]
39			[6-7]		[7-8]	[5-8]	[8-9]	[6-8]	[7-9]	[5-6]	[7-8]	[7-8]
41			[5-7]		[5-6]	[5-8]	inactif 10µM	[4-6]	[7-9]	[5-6]	[6-7]	[7-9]
36			[5-6]		[5-6]	[5-8]	inactif 10µM	[4-7]	[6-8]	[5-6]	[6-7]	[7-9]
49			[6-7]		[6-7]	[6-7]	[7-9]	[5-7]	[10-11]	[6-7]	[7-9]	[8-10]
50			[6-7]		[5-6]	[6-7]	[7-8]	[5-6]	[7/10]	[5-6]	[7-8]	[10-11]
B			[6-7]		[6-7]		[7-8]	[6-7]	[7-8]		[6-8]	[8-9]
54			[6-7]		[5-6]		[6-8]	[5-6]	[7-8]		[6-8]	[7-9]
46			[6-7]		[5-6]		[5-6]	[5-6]	[8/9]		[6-8]	[5-6]

	Bladder			Breast		CNS	Colon	Leukemia	Lung		Prostate	
Example	T24s	T24r	MCF7s	MCF7ras	MCF7 mdr	SK-N-SH neuroblastoma	HT29	K562 /Adr	A427	Calu-1	PC3	DU145
64			[5-7]		[6-7]		[8-9]	[5-6]	[8-10]		[7-8]	[10-13]
51			[6-7]		[6-7]		[6-7]	[5-7]	[7-8]		[6-7]	[7-8]
55			[5-7]		[5-6]		[6-8]	[5-6]	[7-8]		[6-7]	[7-8]
77			[<5]		[5-6]		[5-6]	[<5]	[6-7]		[5-6]	[6-7]
78			[4-5]		[5-6]		[6-7]	[4-6]	[7-9]		[6-7]	[7-8]
52			[5-6]		[5-6]		[6-7]	[5-6]	[6-8]		[6-7]	[7-8]
C			[<5]		[<5]		[5-6]	[4-6]	[6-7]		[5-6]	[6-7]
62			[5-6]		[5-6]		[6-7]	[4-6]	[7-9]		[6-7]	[7-9]
53			[5-7]		[6-7]		[6-8]	[6-8]	[7-9]		[7-8]	[8-10]
63			[6-7]		[6-7]		[7-8]	[6-7]	[8-9]		[7-8]	[8-9]
47			[5-6]		[6-7]		[3-4] ;[7]	[5-7]	[7-8]		[6-7]	[7-9]
D			[<5]		[5-6]		[7-8]	[5-6]	[7-8]		[7]	[7-9]
56			[5-7]		[6-7]		[7-8]	[5-7]	[6-7]		[7-8]	[7-8]
79			[<5]		[5-6]		[6-7]	[5-7]	[8-9]		[7]	[7-8]
58			[<5]		[5-6]		[6-8]	[6-8]	[8-9]		[7-8]	[8-10]
60			[<5]		[5-6]		[7-9]	[6-7]	[9-10]		[8]	[9-10]
68			[<5]		[<5]		[5-7]	[<5]	[7-8]		[6-7]	[6-8]
69			[<5]		[6-7]		[6-7]	[5-6]				[7-8]
70			[6-7]		[5-6]		[6-7]	[5-6]				[8-9]
57			[5-7]				[6-8]	[6-8]	[8-10]			[9-10]
67			[6-7]				[7-9]	[5-8]	[7-9]			[8-9]
48			[5-6]				[6-7]	[5-7]	[7-8]			[7-8]
59	[8-13]	[7-8]	[5-7]				[7-8]	[4-6]	[7-8]		[7-8]	[8-9]
66			[5-7]				[6-8]	[5-6]	[6-8]		[7-8]	[8-10]
39 (HCl salt)	[8-9]	[7-8]	[6-7]				[7-8]	[6-7]	[8-9]		[6-8]	[8-10]
58 (HCl salt)	[8-10]	[8-9]	[5]				[8-9]	[6-7]	[8-9]		[6-8]	[9-10]
67 (HCl salt)	[8-9]	[7-8]	[<5]				[7-8]	[6-7]	[7-9]		[6-7]	[8-10]
66 (HCl salt)	[5-6]	[5-6]	[<5]				[7-8]	[5-6]	[7-8]		[6-7]	[8]
71	[7-8]	[7-8]	[6-7]				[6-7]	[5-7]	[7-8]		[6-7]	[7-8]
64 (HCl salt)							[6]					
61	[7-8]	[6-7]					[6-7]		[5-6]		[5-6]	
43	[>13]	[7-9]					[6-8]	[5-6]	[7-8]		[6-7]	
B (HCl salt)	[8-10]	[7-8]					[7-8]	[6]	[6-8]		[6-7]	
63 (HCl salt)	[9-11]	[7-8]					[7-8]	[6]	[7-8]		[7]	
57 (HCl salt)	[9-10]	[8-9]					[8]	[6-7]	[7-8]		[7-8]	
65 (HCl salt)	[8-9]	[7-8]					[7-8]	[6]	[7-8]		[7]	
44 (HCl salt)	[8-9]	[8-9]					[7-8]	[6-7]	[7-8]		[7]	
44	[7-8]	[7-8]					[6-7]	[5-6]	[6-7]		[6]	
45 (HCl salt)	[12-13]						[7-8]	[6]	[6-7]		[6-7]	
45	[8-9]	[7-8]					[7-8]	[6-7]	[6-7]		[6-7]	
59 (HCl salt)	[9-10]	[8-9]					[8-9]	[6-7]	[8-9]		[7]	
71 (HCl salt)	**19,70**	**10,30**	**46,90**		**556**		**40,60**	**3,92**	**324**		**129**	**17,70**
71 (HCl salt)									**14,50**		**103**	**5,42**
81 ((5S) enantiomer)							inactif 10µM					
81	**0,40**	**0,37**	**32,00**		**459**		**13,50**	**1,69**	**25,10**		**14,50**	**1,34**
81	**4,53**	**2,40**						**119**	**2,02**		**42,80**	**1,58**
82	**2,23**	**2,87**	**48,20**		**333**		**20,90**	**759**	**136**		**30,00**	**6,96**
82	**13,90**	**5,13**						**18,40**	**6,15**		**80,80**	**3,13**
82 ((5S) enantiomer)							inactif 10µM					

	Bladder		Breast			CNS	Colon	Leukemia	Lung		Prostate	
Example	T24s	T24r	MCF7s	MCF7ras	MCF7 mdr	SK-N-SH neuroblastoma	HT29	K562 /Adr	A427	Calu-1	PC3	DU145
86			130		695		42					
87			17		190		16					
98		0,11	10		33		2,10					
95			10		71		8,50					
89			15		188		13					
96			494		1110		32,60					
88			276		276		11,60					
100		0,32	153				2,10					
24		4,48	1070				26,70					
90			312				17					
89			>10000				2950					
99							5,00					
104							8,61					
87							inactif 10µM					
84		0,17					2,50					
91		0,69					11					
92		3,05					21					
E		126					1750					
161		8,89					523					
83		1,58					19					
93		0,10					12					
97		0,17					11					
105		0,30					9,50					
102		0,31					8,30					
101		0,03					1,30					
103		3,00					12					
94		0,004					1,60					
85		21,50					196					
107		0,30					3,73					
106		0,10					3,50					
140		2,93					3,18					
162		0,53					12,20					
F		0,11					1,74					
115		0,11					0,26					
163		1,44					6,00					
117		0,14					0,27					
165		0,53					1,70					
168		3,86					33					
164		0,06					2,70					
167		0,63					12,80					
166		0,61					9,00					
171		0,67					8,20					
16 ((5R) enantiomer)		0,09					2,30					
160	0,66	0,43					5,52	2,40				
72 ((5R) enantiomer)							5,80					
169		0,0005					1,77					
170		0,01					0,85					

COLOR :

■	IC50< 1nM
	1nM< IC50 < 1µM
	1µM< IC50 < 10µM
	inactif ou IC50 > 10µM

Legend : italics => treatment 96h, non italics => treatment 72h

Legend : italics => treatment 96h, non italics => treatment 72h

STOMACH CELL LINE (AGS)

Example	Test 1 IC50 (nM)	Test 2 IC50 (nM)	*Mean IC50 (nM)*	*SD*
9	8,15	19,40	***13,78***	***7,95***
12	60,80	30,90	***45,85***	***21,14***
16 ((5R) enantiomer)	72,40	136,00	***104,20***	***44,97***
19	15,90	11,50	***13,70***	***3,11***
20	42,80	16,70	***29,75***	***18,46***
22	20,70	42,10	***31,40***	***15,13***
24 ((5R) enantiomer)	104,00	104,00	***104,00***	***0,00***
27	865,00	767,00	***816,00***	***69,30***
28	63,70	38,50	***51,10***	***17,82***
31	74,30	127,00	***100,65***	***37,26***
32	203,00	135,00	***169,00***	***48,08***
34	179,50	61,80	***120,65***	***83,23***
40	125,00	162,00	***143,50***	***26,20***
42	36,70	97,70	***67,20***	***43,13***
44 (HCl salt)	267,00	167,00	***217,00***	***70,71***
45	60,10	42,50	***51,30***	***12,45***
49	207,00	138,00	***172,50***	***48,79***
50	115,00	115,00	***115,00***	***0,00***
57	157,00	116,00	***136,50***	***28,99***
59	124,00	71,10	***97,55***	***37,41***
72 ((5R) enantiomer)	61,20	92,50	***76,85***	***22,13***
75 (HCl salt)	60,00	37,70	***48,85***	***15,77***
81	22,50	22,50	***22,50***	***0,00***
82	334,00	167,00	***250,50***	***118,09***
82 (free base)	31,60	31,50	***31,55***	***0,07***
83	39,20	63,70	***51,45***	***17,32***
88	44,30	44,30	***44,30***	***0,00***
89	83,00	82,90	***82,95***	***0,07***
90	25,90	25,90	***25,90***	***0,00***
91	39,80	39,80	***39,80***	***0,00***
92	238,00	238,00	***238,00***	***0,00***
93	115,00	135,00	***125,00***	***14,14***
94	12,90	19,80	***16,35***	***4,88***
95	63,80	63,80	***63,80***	***0,00***
96	22,30	22,50	***22,40***	***0,14***
97	41,60	78,00	***59,80***	***25,70***
98	16,40	16,40	***16,40***	***0,00***
100	24,30	24,30	***24,30***	***0,00***
102	6,52	2,45	***4,49***	***2,88***
103	23,00	12,80	***17,90***	***7,21***
104	57,00	59,70	***58,35***	***1,91***
107	113,00	130,00	***121,50***	***12,02***
115	42,90	56,10	***49,50***	***9,33***
117	78,50	30,00	***54,30***	***34,30***
140	9,73	6,64	***8,19***	***2,18***
161	394,00	394,00	***394,00***	***0,00***
162	227,00	410,00	***318,50***	***129,40***
163	238,00	328,00	***283,00***	***63,64***
164	18,80	25,30	***22,05***	***4,60***
166	58,60	111,00	***84,80***	***37,05***
167	97,70	134,00	***115,85***	***25,67***
168	186,00	231,00	***208,50***	***31,82***
169	29,50	12,70	***21,10***	***11,88***
171	194,00	333,00	***263,50***	***98,29***
174	206,00	196,00	***201,00***	***7,07***
175	105,00	174,00	***139,50***	***48,79***
176 (HCl salt)	99,30	48,10	***73,70***	***36,20***
177	203,00	152,00	***177,50***	***36,06***
178	205,00	280,00	***242,50***	***53,03***
179	491,00	489,00	***490,00***	***1,41***
180	78,90	48,20	***63,55***	***21,71***
181	569,00	671,00	***620,00***	***72,12***
182	324,00	714,00	***519,00***	***275,77***
185	42,50	18,50	***30,50***	***16,97***

SD = standard deviation

OVARY CELL LINE (SKOV-3)

Examples	Test 1 IC50 (nM)	Test 2 IC50 (nM)	*Mean IC 50 (nM)*	*SD*
9	16,20	11,40	***13,80***	***3,39***
12	14,70	7,80	***11,30***	***4,90***
15	131,00	122,00	***126,50***	***6,36***
16	1,84	5,66	***3,75***	***2,70***
19	5,47	2,47	***3,97***	***2,12***
20	25,50	11,00	***18,25***	***10,25***
22	10,00	7,09	***8,55***	***2,06***
24	20,20	13,60	***16,90***	***4,67***
27	733,00	681,00	***707,00***	***36,77***
28	18,80	7,26	***13,03***	***8,16***
31	18,10	14,90	***16,50***	***2,26***
32	163,00	102,00	***132,50***	***43,13***
34	83,00	128,00	***105,50***	***31,82***
40	160,00	85,10	***122,55***	***52,96***
42	23,00	10,00	***16,50***	***9,19***
44	94,40	45,30	***69,85***	***34,72***
45	23,20	50,70	***36,95***	***19,45***
49	15,50	10,90	***13,20***	***3,25***
50	27,80	16,50	***22,15***	***7,99***
57	19,50	11,70	***15,60***	***5,52***
59	22,80	26,50	***24,65***	***2,62***
72 ((5R) enantiomer)	12,20	11,40	***11,80***	***0,57***
75 (HCl salt)	21,50	43,60	***32,55***	***15,63***
81	2,92	5,32	***4,12***	***1,70***
82	16,30	33,70	***25,00***	***12,30***
82 (free base)	9,08	10,90	***9,99***	***1,29***
83	8,09	10,70	***9,40***	***1,85***
88	14,30	8,41	***11,36***	***4,16***
89	16,50	14,10	***15,30***	***1,70***
90	7,05	6,64	***6,85***	***0,29***
91	20,70	14,50	***17,60***	***4,38***
92	72,60	39,10	***55,85***	***23,69***
93	7,19	8,24	***7,72***	***0,74***
94	3,31	2,54	***2,93***	***0,54***
95	4,84	10,90	***7,87***	***4,29***
96	22,50	14,10	***18,30***	***5,94***
97	5,71	3,54	***4,63***	***1,53***
98	2,70	6,24	***4,47***	***2,50***
100	5,57	6,95	***6,26***	***0,98***
102	4,30	2,01	***3,16***	***1,62***
103	5,06	6,97	***6,02***	***1,35***
104	14,40	9,33	***11,87***	***3,59***
107	7,82	4,11	***5,97***	***2,62***
115	2,75	1,32	***2,04***	***1,01***
117	8,15	10,90	***9,53***	***1,94***
140	6,16	9,73	***7,95***	***2,52***
161	191,00	161,00	***176,00***	***21,21***
162	8,63	9,42	***9,03***	***0,56***
163	19,20	9,91	***14,56***	***6,57***
164	7,69	6,61	***7,15***	***0,76***
166	15,20	20,30	***17,75***	***3,61***
167	15,90	12,70	***14,30***	***2,26***
168	72,80	81,30	***77,05***	***6,01***
169	7,92	10,30	***9,11***	***1,68***
171	9,29	15,40	***12,35***	***4,32***
174	17,30	11,60	***14,45***	***4,03***
175	23,80	27,20	***25,50***	***2,40***
176	11,40	11,70	***11,55***	***0,21***
177	11,90	19,10	***15,50***	***5,09***
178	37,60	19,50	***28,55***	***12,80***
179	17,00	38,10	***27,55***	***14,92***
180	38,80	78,90	***58,85***	***28,35***
181	38,40	97,00	***67,70***	***41,44***
182	11,10	20,70	***15,90***	***6,79***
183	598,00	601,00	***599,50***	***2,12***
185	10,80	19,10	***14,95***	***5,87***

SD = standard deviation

MELANOMA CELL LINE (UZG 4)

Example	Test 1 IC50 (nM)	Test 2 IC50 (nM)	*Mean IC50 (nM)*	*SD*
9	289,00	245,00	*267,00*	*31,11*
12	169,00	130,00	*149,50*	*27,58*
16 ((5R) enantiomer)	57,50	46,50	*52,00*	*7,78*
19	220,00	210,00	*215,00*	*7,07*
20	292,00	160,00	*226,00*	*93,34*
22	167,00	142,00	*154,50*	*17,68*
24 ((5R) enantiomer)	175,00	187,00	*181,00*	*8,49*
28	138,00	160,00	*149,00*	*15,56*
31	344,00	720,00	*532,00*	*265,87*
34	767,00	651,00	*709,00*	*82,02*
39 (HCl salt)	869,00	863,00	*866,00*	*4,24*
42	247,00	175,00	*211,00*	*50,91*
44	700,00	615,00	*657,50*	*60,10*
45	354,00	293,00	*323,50*	*43,13*
49	193,00	302,00	*247,50*	*77,07*
50	420,00	248,00	*334,00*	*121,62*
57	295,00	335,00	*315,00*	*28,28*
59	424,00	365,00	*394,50*	*41,72*
59 (HCl salt)	522,00	393,00	*457,50*	*91,22*
72 ((5R) enantiomer)	86,30	106,00	*96,15*	*13,93*
75	640,00	807,00	*723,50*	*118,09*
81	127,00	104,00	*115,50*	*16,26*
82	379,00	429,00	*404,00*	*35,36*
82 (free base)	223,00	114,00	*168,50*	*77,07*
83	114,00	176,00	*145,00*	*43,84*
88	269,00	220,00	*244,50*	*34,65*
89	21,90	37,80	*29,85*	*11,24*
90	186,00	147,00	*166,50*	*27,58*
91	163,00	224,00	*193,50*	*43,13*
92	265,00	305,00	*285,00*	*28,28*
93	319,00	259,00	*289,00*	*42,43*
94	55,10	68,40	*61,75*	*9,40*
95	109,00	173,00	*141,00*	*45,25*
96	324,00	377,00	*350,50*	*37,48*
97	290,00	217,00	*253,50*	*51,62*
98	87,50	69,00	*78,25*	*13,08*
100	136,00	121,00	*128,50*	*10,61*
102	80,50	55,20	*67,85*	*17,89*
103	262,00	128,00	*195,00*	*94,75*
104	190,00	155,00	*172,50*	*24,75*
107	110,00	149,00	*129,50*	*27,58*
115	37,80	47,30	*42,55*	*6,72*
117	49,10	44,90	*47,00*	*2,97*
140	147,00	184,00	*165,50*	*26,16*
161	612,00	641,00	*626,50*	*20,51*
162	318,00	212,00	*265,00*	*74,95*
163	199,00	163,00	*181,00*	*25,46*
164	119,00	95,30	*107,15*	*16,76*
166	245,00	193,00	*219,00*	*36,77*
167	201,00	216,00	*208,50*	*10,61*
168	703,00	601,00	*652,00*	*72,12*
169	46,00	52,00	*49,00*	*4,24*
171	69,80	43,80	*56,80*	*18,38*
174	265,00	261,00	*263,00*	*2,83*
175	280,00	384,00	*332,00*	*73,54*
176 (HCl salt)	346,00	406,00	*376,00*	*42,43*
178	506,00	442,00	*474,00*	*45,25*
179	440,00	533,00	*486,50*	*65,76*
180	524,00	484,00	*504,00*	*28,28*
181	544,00	659,00	*601,50*	*81,32*

SD = standard deviation

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Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays

Tim Mosmann

DNAX Research Institute of Molecular and Cellular Biology, Inc., 1450 Page Mill Road, Palo Alto, CA 94304, U.S.A.

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A tetrazolium salt has been used to develop a quantitative colorimetric assay for mammalian cell survival and proliferation. The assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cells. This method can therefore be used to measure cytotoxicity, proliferation or activation. The results can be read on a multiwell scanning spectrophotometer (ELISA reader) and show a high degree of precision. No washing steps are used in the assay. The main advantages of the colorimetric assay are its rapidity and precision, and the lack of any radioisotope. We have used the assay to measure proliferative lymphokines, mitogen stimulations and complement-mediated lysis.

Key words: *lymphokine assays – proliferation assays – colorimetric assay – tetrazolium – TCGF*

Introduction

Many biological assays require the measurement of surviving and/or proliferating mammalian cells. This can be achieved by several methods, e.g., counting cells that include/exclude a dye, measuring released ^{51}Cr-labeled protein after cell lysis, and measuring incorporation of radioactive nucleotides ([^{3}H]thymidine or [^{125}I]iododeoxyuridine) during cell proliferation. The radioactive method can be partially automated and can handle moderately large numbers of samples, but even with these methods, it is difficult to process thousands of assay points per day. In our current research we assay many samples of various lymphokines that induce cell proliferation, and so we required a rapid and quantitative assay capable of handling large numbers of samples.

Viable cells could be measured by using any of several staining methods, but we wished to avoid any washing steps that would increase processing time and sample variation. Multiwell scanning spectrophotometers (ELISA readers) can measure large numbers of samples with a high degree of precision, and so we investigated the possibility of using a color reaction as a measure of viable cell number. Ideally, a

colorimetric assay for living cells should utilize a colorless substrate that is modified to a colored product by any living cell, but not by dead cells or tissue culture medium. Tetrazolium salts are attractive candidates for this purpose, since they measure the activity of various dehydrogenase enzymes (Slater et al., 1963). The tetrazolium ring is cleaved in active mitochondria, and so the reaction occurs only in living cells.

We have developed a rapid colorimetric assay, based on the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), that measures only living cells and can be read on a scanning multiwell spectrophotometer (ELISA reader). This assay is versatile and quantitative, and we consider it a significant advance over traditional techniques for several commonly used proliferation and cytotoxicity assays.

Materials and Methods

Cell lines

The EL_4G^- mouse lymphoma cell line was obtained from G. Carlson, and subclone $EL_4.3$ was selected for growth in 6-thioguanine. Another subline of EL_4, designated EL_4E2, was obtained from V. Paetkau. The EL_4E2 subline produces large quantities of interleukin 2 when stimulated with phorbol myristate acetate (Farrar et al., 1980). A continuous line of mouse T cells, A70 13/13, was derived in the author's laboratory at the University of Alberta. All cells were grown in RPMI 1640 supplemented with 50 μM 2-mercaptoethanol and 5–10% fetal bovine serum, in a 6% CO_2 atmosphere.

Colorimetric MTT (tetrazolium) assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma catalog no. M2128) was dissolved in PBS at 5 mg/ml and filtered to sterilize and remove a small amount of insoluble residue present in some batches of MTT. At the times indicated below, stock MTT solution (10 μl per 100 μl medium) was added to all wells of an assay, and plates were incubated at 37°C for 4 h. Acid-isopropanol (100 μl of 0.04 N HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on a Dynatech MR580 Microelisa reader, using a test wavelength of 570 nm, a reference wavelength of 630 nm, and a calibration setting of 1.99 (or 1.00 if the samples were strongly colored). Plates were normally read within 1 h of adding the isopropanol.

Interleukin 2 assay

Interleukin 2 (IL2) was derived from phorbol-myristate-acetate stimulated EL_4E2 cells (Farrar et al., 1980). The IL2-dependent T cell line A70 13/13 was used as an indicator cell. Doubling dilutions of IL2 were prepared in 96-well trays using growth medium as diluent. T cells (2000 per well) were then added, with a final volume of 0.1 ml per well. At 48 h, proliferation was measured by the MTT colorimetric assay.

Mitogen-induced proliferation of spleen cells

BALB/c mouse spleen cells were stimulated in 0.1 ml at 10^6 cells/well with varying concentrations of *Salmonella typhosa* lipopolysaccharide (LPS; Sigma) or concanavalin A (Con A; Calbiochem) and assayed at 3 days for proliferation using both colorimetric MTT and [^{3}H]thymidine incorporation assays. For the radioactive assay, 0.001 mCi [^{3}H]thymidine was added to each well, and after 4 h at 37°C the cells were harvested using a PHD cell harvester (Cambridge Instruments, Cambridge, MA).

Computer processing

Readings from the Dynatech MR580 Microelisa reader were transferred directly to an Apple II computer, using a program that saved the results to a diskette and printed the OD values in a 96-well format that matched the original plate. Additional programs were written to process the data stored on diskettes. We now have programs to plot results, calculate and plot means and standard deviations, identify wells above a chosen threshold, and calculate units of growth factor. These programs are available on request.

Results

In preliminary experiments, we tested several tetrazolium salts by incubation with cells for several hours. The most promising reagent was MTT, a pale yellow substrate that produced a dark blue formazan product when incubated with live cells. The MTT formazan reaction product was only partially soluble in the medium, and so an alcohol was used to dissolve the formazan and produce a homogeneous solution suitable for measurement of optical density. Initially, ethanol was used for this purpose, but some precipitation of serum proteins occassionally occurred in the acid-alcohol mixture. Several other organic solvents were tested, and isopropanol was found to be the most suitable solvent. Normal tissue culture medium has a variable color due to pH changes and the red form of phenol red interfered at the wavelength most suitable for blue MTT formazan measurement. To minimize this interference, we converted the phenol red to the fully acidic, yellow form at the end of the assay.

Our final procedure was to add 0.01 ml MTT (5 mg/ml in phosphate-buffered saline) to 0.1 ml cells in growth medium. After 4 h at 37°C for MTT cleavage, the formazan product was solubilized by the addition of 0.1 ml 0.04 N HCl in isopropanol. Optical density was measured on a Dynatech MR 580 plate reader, using a reference wavelength of 630 nm and a test wavelength of 570 nm.

EL_4.3 lymphoma cells were used to test the relationship between cell number and the amount of MTT formazan generated. The results in Fig. 1 show that the absorbance is directly proportional to the number of cells. This linearity extends over almost the entire range tested, from 50,000 to 200 cells/well. In addition, these results indicate that the assay is capable of detecting very small numbers of living cells (e.g., 200). The actual cells do not absorb significantly, even at a concentration of 1×10^6 cells/ml.

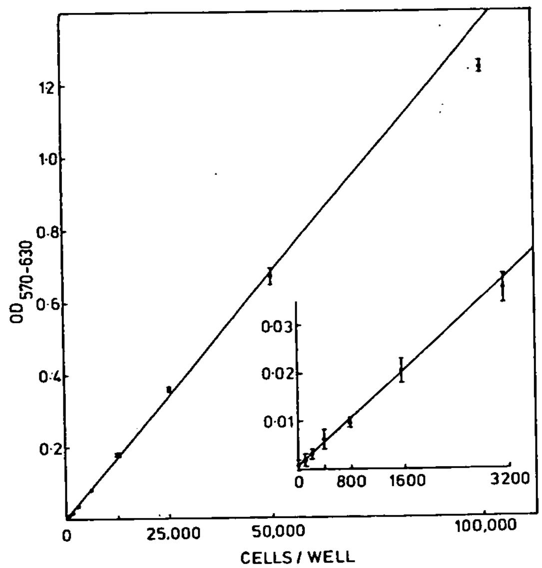

Fig. 1. Linearity of the MTT assay. EL_4.3 cells were plated out in doubling dilutions in 0.1 ml growth medium (RPMI 1640 + 10% fetal bovine serum) in 96-well flat-bottomed trays (Falcon), starting at 10^5 cells/well. MTT (0.01 ml of 5 mg/ml stock) was added immediately to all wells, and the plates were incubated at 37°C for 3 h, developed and measured. Each point shows the mean and standard deviation of 4 replicates. The straight line plotted is the best fit line calculated using all points from 100 to 50,000 cells/well.

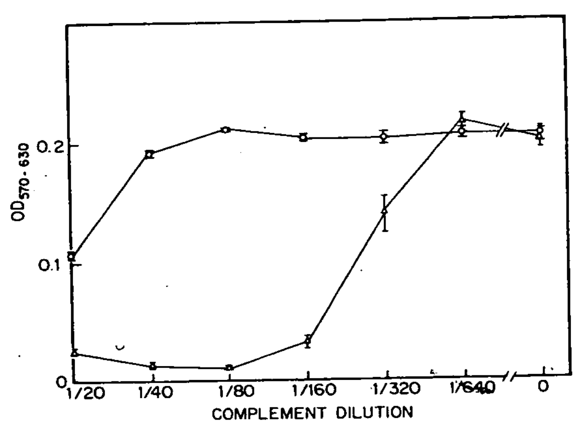

Fig. 2. Living cells are required for MTT cleavage. EL_4.3 cells were treated with anti-Thy1.2 (Cedarlane), and then treated cells and untreated controls were added to rabbit complement dilutions in a 96-well tray. After 30 min incubation at 37°C, MTT was added to all wells, and after another 4 h the plates were developed and read. Each point shows the mean and standard deviation of 4 replicates. Δ, anti-Thy1.2-treated; O, untreated.

In many assays, dead cells will be present, and so it was important to determine if recently killed cells were positive or negative in the assay. Fig. 2 shows that only live cells actively cleave MTT, while dead cells are almost completely negative even immediately after complement-mediated lysis. These results suggested that living cells with active mitochondria are required to generate a strong signal, and raised the possibility that the amount of formazan generated per cell would depend on the level of energy metabolism in the cell. To test this, we measured formazan generation by metabolically inactive cells (red blood cells), resting cells (spleen cells) and activated cells (concanavalin A-stimulated lymphocytes). Fig. 3a shows that neither chicken nor sheep red blood cells cleave MTT to a significant extent, and neither red cell type interferes significantly in the assay, up to concentrations of 2×10^6 cells/ml. Fig. 3b shows that Con A-activated lymphocytes produce approximately 10 times as much formazan per cell as their normal counterparts.

A continuous line of interleukin 2 (IL2)-dependent T cells (A70 13/13), previously established in the author's laboratory at the University of Alberta, was used as

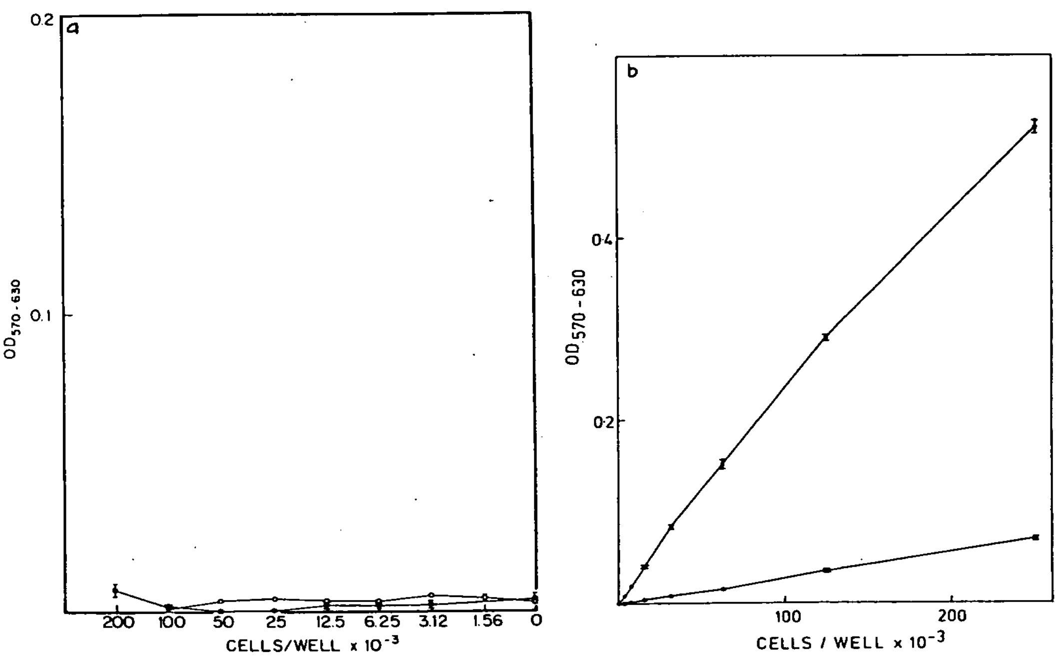

Fig. 3. MTT cleavage by erythrocytes and normal and activated lymphocytes. a: chicken and sheep erythrocytes were incubated in 0.1 ml medium with MTT for 3 h at 37°C. The plates were then developed and read. Means and standard deviations of 3 replicates per point are shown. ●, chicken erythrocytes; O, sheep erythrocytes. b: mouse spleen cells were stimulated with 2 μg/ml concanavalin A for 48 h, and then the Con A-activated lymphocytes and normal lymphocytes were plated in doubling dilutions in 96-well flat-bottomed trays. MTT was added immediately, and after 2 h, the plates were developed and read. The means and standard deviations of 3 replicates per point are shown: ●, Con A-activated lymphocytes; O, normal lymphocytes.

the target cell for an IL2 assay. This assay was used to test 4 parameters of the colorimetric reaction – the length of exposure of cells to IL2, the duration of MTT treatment, the concentration of MTT used, and the number of test cells added to the assay.

Fig. 4 shows the effect of varying the time of incubation with MTT. The signal increased almost linearly from 1/2 to 2 h, but increased at a lesser rate from 2 to 4 h. In similar experiments, the concentration of MTT and cell number were optimized for the cell lines used in our studies (results not shown). The formazan generated was approximately proportional to the MTT concentration at low concentrations, and reached a plateau at about 0.45 mg/ml MTT. The formazan generated was also proportional to the number of cells at high IL2 concentrations, but the amount of factor required to produce 50% stimulation was increased at higher target cell concentrations. The assay could be read at 1, 2 or 3 days, but the apparent titer of the IL2 declined with increasing incubation time, probably due to depletion of the growth factor during cell growth. Our optimum values for these 4 parameters may need modifying for other assays, but in general, we have found that widely differing cell lines require only minimal changes.

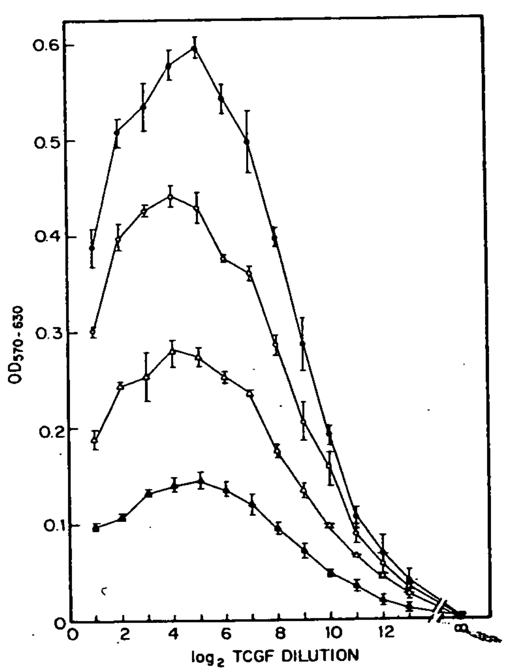

Fig. 4. Duration of MTT incubation. Interleukin 2 dilutions were assayed on A70 13/13 T cells. MTT was added at 44, 46, 47 and $47\frac{1}{2}$ h, to sets of 3 rows each, and at 48 h, all wells were developed and read. Optical density readings were measured relative to control wells containing medium, cells and MTT but no growth factor. The means and standard deviations of 3 replicates per point are shown. ▲, 1/2 h; △, 1 h; ○, 2 h; ●, 4 h.

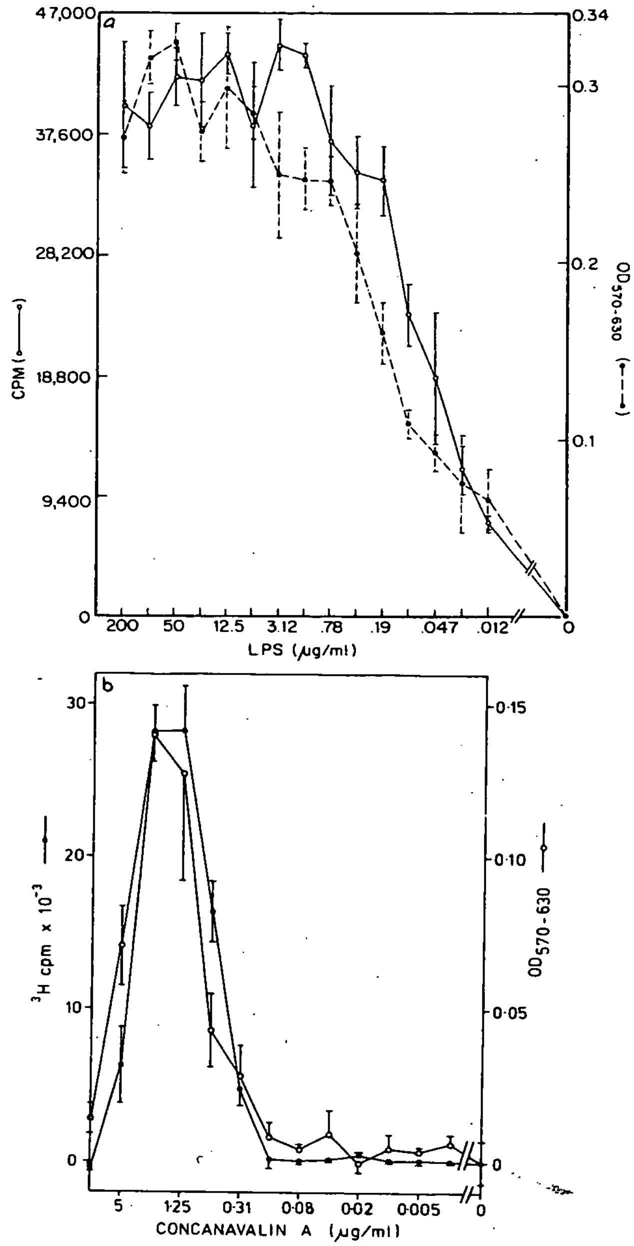

Fig. 5. Mitogen-induced proliferation of spleen cells. Spleen cells were stimulated for 3 days with varying concentrations of LPS and Con A and proliferation was measured using both colorimetric and radioactive assays. Results are shown as the means and standard deviations of 4 replicates per point. Background values, obtained from wells with cells but no mitogen, were subtracted from all points. a: LPS stimulation. b: Con A stimulation.

The results of the colorimetric assay with cloned cell lines were very encouraging, and so we explored the utility of the assay in more complex systems: the lymphocyte proliferative responses to the mitogens Con A and LPS. Since many cell types are present in the cell populations normally used for such proliferations, it was conceivable that certain cell types would generate abnormally large or small signals. Accordingly, we compared the colorimetric assay to a [^{3}H]thymidine incorporation assay for both Con A and LPS responses of normal mouse spleen cells.

Stimulation with both mitogens was measured effectively by both assays (Fig. 5). LPS stimulated cells over an extended concentration range, whereas the titration curve for Con A showed a narrow optimum, with little or no proliferation at high or low concentrations. The colorimetric and radioactive assays showed excellent agreement for Con A stimulations, and showed a small difference between the endpoint of LPS stimulations.

Activated macrophages produce more formazan product from nitroblue tetrazolium than do non-activated macrophages (Baehner et al., 1976), and so we measured MTT formazan production after LPS activation of a macrophage-like cell line, P388D1 (Lachman et al., 1977). No increase in MTT formazan production was seen after stimulation with a wide range of LPS concentrations, and P388D1 cells did not produce an unusual amount of MTT formazan (results not shown).

Discussion

The cleavage of MTT has several desirable properties for assaying cell survival and proliferation. MTT is cleaved by all living, metabolically active cells that we have tested, but not by dead cells or erythrocytes. The amount of formazan generated is directly proportional to the cell number over a wide range, using a homogeneous cell population. Activated cells produce more formazan than resting cells, which could allow the measurement of activation even in the absence of proliferation. These properties are all consistent with the cleavage of MTT only by active mitochondria.

The main advantage of the colorimetric assay is the speed with which samples can be processed. The substrate does not interfere with measurement of the product, and we have found conditions in which components of the medium do not interfere. This allows the assay to be read with no removal or washing steps, which increases the speed of the assay and helps to minimize variability between samples. The final stages of the assay (adding the MTT, reading the plate and printing the data) take much less time than setting up the assay (mixing cells and growth factor dilutions). The assay can be read a few minutes after the addition of acid-isopropanol, and the color is stable for a few hours at room temperature. The results are also apparent visually, which is very useful if rapid qualitative results are required.

The colorimetric assay measures the number and activity of living cells at the end of the assay, whereas [^{3}H]thymidine incorporation measures the number of cells synthesizing DNA during the last few hours of the assay. So the colorimetric assay correlates well with visual examination of the cells at the end of the assay (Kappler

et al., 1981) but these 2 assays can potentially differ from radioactive nucleotide incorporation methods. This should be kept in mind for specific applications, e.g., distinguishing between death, survival and proliferation. In practice, we have not seen large differences between the colorimetric assay, radioisotope assay or visual inspection of the wells.

The only additional reagents used in the assay are MTT, isopropanol, and HCl. No radioisotopes are used, and no scintillation counter or gamma-counter is needed. This advantage is partially offset by the requirement for a plate reader, but the high scanning rate of typical machines (e.g., $1\frac{1}{2}$ min per 96 wells) allows a single plate reader to handle very large numbers of samples.

The colorimetric assay shares with the radioisotope assays the advantages of precise quantitation and compatibility with computer analysis programs. Since the colorimetric assay is so rapid, large amounts of data can be generated, and some form of computer processing is very desirable. We have set up programs for calculating means and standard deviations, plotting curves, and calculating units of growth factor in the original sample (using a linear interpolation to calculate the exact dilution at which stimulation is a preset value, e.g., 25% of the maximum plateau stimulation). These programs are written for an Apple II computer, and are available on request.

The reduction of MTT to a formazan product appears to be carried out by all the cell types we have examined. These include mitogen stimulated T and B cells, myeloma, T lymphoma and macrophage-like tumor cell lines, as well as various IL2-dependent T cell lines. This suggests that the colorimetric MTT assay may have very wide applicability for measuring survival and/or proliferation of various cells and can potentially be applied to any assay in which living cells must be distinguished from dead cells or a lack of cells. The results in Fig. 2 show that dead cells are unable to cleave MTT within 30 min of complement-mediated lysis. This indicates that the assay also has potential value for quantitative and rapid measurement of cell death, e.g., in HLA typing. The MTT assay may also be applicable to the assay of cytotoxic T lymphocytes, although the signal generated by the CTL population could mask the signal from the target population at high effector : target ratios.

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Evaluation of a Soluble Tetrazolium/Formazan Assay for Cell Growth and Drug Sensitivity in Culture Using Human and Other Tumor Cell Lines[1]

Dominic A. Scudiero,[2] Robert H. Shoemaker, Kenneth D. Paull, Anne Monks, Si bhan Tierney, Thomas H. Nofziger, Michael J. Currens, Donna Seniff, and Michael R. Boyd

Program Resources, Inc., National Cancer Institute-Frederick Cancer Research Facility, Frederick, Maryland 21701 [D. A. S., A. M., S. T., M. C., T. N., D. S.], and Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland 20892 [R. S., K. P., M. B.]

ABSTRACT

We have previously described the application of an automated microculture tetrazolium assay (MTA) involving dimethyl sulfoxide solubilization of cellular-generated 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-formazan to the *in vitro* assessment of drug effects on cell growth (M. C. Alley *et al.*, Proc. Am. Assoc. Cancer Res., *27:* 389, 1986; M. C. Alley *et al.*, Cancer Res. *48:* 589–601, 1988). There are several inherent disadvantages of this assay, including the safety hazard of personnel exposure to large quantities of dimethyl sulfoxide, the deleterious effects of this solvent on laboratory equipment, and the inefficient metabolism of MTT by some human cell lines. Recognition of these limitations prompted development of possible alternative MTAs utilizing a different tetrazolium reagent, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT), which is metabolically reduced in viable cells to a water-soluble formazan product. This reagent allows direct absorbance readings, therefore eliminating a solubilization step and shortening the microculture growth assay procedure. Most human tumor cell lines examined metabolized XTT less efficiently than MTT; however, the addition of phenazine methosulfate (PMS) markedly enhanced cellular reduction of XTT. In the presence of PMS, the XTT reagent yielded usable absorbance values for growth and drug sensitivity evaluations with a variety of cell lines. Depending on the metabolic reductive capacity of a given cell line, the optimal conditions for a 4-h XTT incubation assay were 50 μg of XTT and 0.15 to 0.4 μg of PMS per well. Drug profiles obtained with representative human tumor cell lines for several standard compounds utilizing the XTT-PMS methodology were similar to the profiles obtained with MTT. Addition of PMS appeared to have little effect on the metabolism of MTT. The new XTT reagent thus provides for a simplified, *in vitro* cell growth assay with possible applicability to a variety of problems in cellular pharmacology and biology. However, the MTA using the XTT reagent still shares many of the limitations and potential pitfalls of MTT or other tetrazolium-based assays.

INTRODUCTION

The metabolic reduction of soluble tetrazolium salts to insoluble colored formazans has been exploited for many years for histochemical localization of enzyme activities (1, 2). In one of the earliest efforts to develop a practical *in vitro* drug sensitivity test, Black and Speer (3) utilized a tetrazolium/formazan method to assess inhibition of dehydrogenase activity by cancer chemotherapeutic drugs in slices of excised tissue. As an *in situ* vital staining process this phenomenon has also been used for identifying viable colonies of mammalian cells in soft agar culture (4) and for facilitating *in vitro* drug sensitivity assays with human tumor cell populations in primary culture (5). Mosmann (6) described a tetrazolium-based assay which allowed rapid measurement of growth of lymphoid cell populations and their response to lymphokines. Recent reports from our laboratories (7, 8) and others (9, 10) have described modifications of Mosmann's procedure for *in vitro* assay of tumor cell response to chemotherapeutic agents. We have found that this MTA[3] approach allows reproducible estimates of drug sensitivity in a variety of human and other tumor cell lines. Moreover, because of its microscale and potential for automation, the MTA is one of several assays under consideration by the National Cancer Institute for potential application to a large-scale antitumor drug-screening program (7, 8).

The previously described MTA (7, 8) requires DMSO solubilization of MTT-formazan generated by cellular reduction of the MTT tetrazolium reagent. This step is not only laborious, but also may risk exposure of laboratory personnel to large quantities of potentially hazardous solutions in DMSO. Frequent DMSO exposure also produces deleterious effects upon some laboratory equipment. Therefore, to allow the investigation of a simplified MTA and to address potential problems associated with solvent handling, a series of new tetraz lium salts have been developed which, upon metabolic reduction by viable cells, yield aqueous-soluble formazans (11). In this paper we describe the development of one such tetrazolium salt (XTT) and its application to the MTA.

MATERIALS AND METHODS

Cell Lines and Culture. Cell lines (Table 1) were maintained as stocks in RPMI 1640 (Quality Biological, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Sterile Systems, Logan, UT) and 2 mM L-glutamine (Central Medium Laboratory, NCI-FCRF). Cell cultures were passaged once or twice weekly using trypsin-EDTA (Central Medium Laboratory, NCI-FCRF) to detach the cells from their culture flasks.

Drugs. All experimental agents were obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, DCT, NCI. Crystalline stock materials were stored at −70°C and solubilized in 100% DMSO. Compounds were diluted into complete medium (RPMI 1640 plus fetal bovine serum) plus 0.5% DMSO before addition to cell cultures.

MTT-Microculture Tetrazolium Assay. Cellular growth in the presence or absence of experimental agents was determined using the previously described MTT-microculture tetrazolium assay (7, 8). Briefly, rapidly growing cells were harvested, counted, and inoculated at the appropriate concentrations (100-μl volume) into 96-well microtiter plates using a multichannel pipet. After 24 h, drugs were applied (100-μl volume) to triplicate culture wells, and cultures were incubated for 6 days at 37°C. MTT (Sigma, St. Louis, MO) was prepared at 5 mg/ml in PBS (Dulbecco and Vogt formulation, without calcium and magnesium; Quality Biological, Gaithersburg, MD) and stored at 4°C. On Day 7, MTT was diluted 1 to 5 in medium without serum (in the MTA described in Refs. 7 and 8, MTT was diluted in complete medium containing 10% fetal bovine serum), and 50 μl were added to microculture wells. After 4-h incubation at 37°C, 250 μl were removed from

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[2] To whom requests for reprints should be addressed, at Building 539, National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD 21701.

[3] The abbreviations used are: MTA, microculture tetrazolium assay; DMSO, dimethyl sulfoxide; MEN, menadione; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PMS, phenazine methosulfate; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide, inner salt, sodium salt; IC_{50}, 50% inhibitory concentration; NCI, National Cancer Institute; FCRF, Frederick Cancer Research Facility; DCT, Division of Cancer Treatment.

Table 1 *Cell strains used in this study*

Cell line	Origin	Source	MTT/XTT absorbance[a] with the following PMS concentrations[b]: MTT, 0.0 mM	XTT 0.0 mM	XTT 0.001 mM	XTT 0.01 mM	XTT 0.0250 mM
H23	Lung adenocarcinoma	a[c]	1.176	0.105	0.115	0.492	1.224
H322	Lung bronchioloalveolar carcinoma	a[c]	0.738	0.242	0.256	0.561	1.337
H324	Lung adenocarcinoma	a[c]	0.492	0.031	0.052	0.710	1.110
H358	Lung bronchioloalveolar carcinoma	a[c]	0.545	0.077	0.081	0.266	0.637
H460	Lung large cell carcinoma	a[c]	2.580	0.288	0.342	1.688	2.830
A549	Lung adenocarcinoma	b[d]	2.700	0.233	0.356	2.360	2.804
LOX	Malignant melanoma	c[e]	1.030	0.201	0.266	1.489	2.279
HT-29	Colon adenocarcinoma	b[d]	1.914	0.201	0.196	1.014	1.555
MCF-7	Breast adenocarcinoma	d[f]	1.110	0.086	0.093	0.374	0.828
CCD-19 LU	Lung fibroblast	b[d]	0.356	0.109	0.120	0.369	1.090
MCR-5	Lung fibroblast	b[d]	0.326	0.186	0.211	0.300	0.695
W138	Lung fibroblast	b[d]	0.257	0.045	0.092	0.317	0.747
P388	Murine leukemia	e[g]	0.674	0.101	0.174	0.426	1.262
	Background ($n = 13$)		0.023 ± 0.006[h]	0.175 ± 0.031	0.186 ± 0.057	0.204 ± 0.034	0.250 ± 0.015

[a] Data represent average absorbance minus background from triplicate wells. Cells were inoculated at 1250 cells/well. Culture duration was for 7 days, and MTT or XTT incubation for 4 h at 37°C.
[b] PMS concentration: 0.001 mM = 0.015 μg/well; 0.01 mM = 0.15 μg/well; 0.025 mM = 0.38 μg/well.
[c] Supplied by Dr. A. Gazdar, Navy Medical Oncology Branch, Division of Cancer Treatment, NCI, Bethesda, MD.
[d] Obtained from the American Type Culture Collection, Rockville, MD.
[e] Supplied by Dr. O. Fodstad, Norwegian Radium Hospital, Oslo, Norway.
[f] Supplied by Dr. K. Cowan, Clinical Pharmacology Branch, Division of Cancer Treatment, NCI, Bethesda, MD.
[g] Supplied by the NCI, DCT Tumor Repository, NCI-FCRF, Frederick, MD.
[h] Mean ± SD of 39 triplicate well background measurements.

each well, and 150 μl of 100% DMSO were added to solubilize the MTT-formazan product. After thorough mixing with a mechanical plate mixer, absorbance at 540 nm was measured with a Dynatech Model MR 600 mciroplate reader.

XTT-Microculture Tetrazolium Assay. The new tetrazolium reagent (XTT) was designed to yield a suitably colored, aqueous-soluble, nontoxic formazan upon metabolic reduction by viable cells. The chemical structures of MTT, XTT, and their respective formazan reduction products are shown in Fig. 1. The presence of two sulfonic acid groups in XTT is the key to its aqueous solubility in both the tetrazolium ion form and the formazan form. XTT has a single net negative charge at physiological pH, and bioreduction of the central positively charged tetrazolium nucleus increases the net charge to two. The corresponding reduction of MTT reduces the net positive charge to zero, and thus the MTT formazan is quite insoluble. For the present investigations, XTT was obtained by a synthetic procedure described elsewhere (11). The XTT reagent is now available from at least one commercial supplier (Polysciences, Warrington, PA).

The XTT-assay methodology was essentially the same as that described using the MTT reagent with the following modifications: XTT was prepared at 1 mg/ml in prewarmed (37°C) medium without serum. PMS (Sigma, St. Louis, MO; Catalogue No. P9625) was prepared at 5 mM (1.53 mg/ml) in PBS. The 5 mM PMS solution was stable at 4°C for at least 3 mo. MEN (Sigma; Catalogue No. M5625) was prepared at 10 mM (1.72 mg/ml) in acetone. MEN was prepared fresh immediately before use. Fresh XTT and PMS were mixed together at the appropriate concentrations. For a 0.025 mM PMS-XTT solution, 25 μl of the stock 5 mM PMS were added per 5 ml of XTT (1 mg/ml). Fifty μl of this mixture (final concentration, 50 μg of XTT and 0.38 μg of PMS per well) were added to each well on Day 7 after cell inoculation. For experiments designed to examine various PMS concentrations, 5 mM PMS was diluted in PBS before addition to the 1-mg/ml solution of XTT. After an appropriate incubation at 37°C (4 h unless otherwise indicated), the plates were mixed on a mechanical plate shaker, and absorbance at 450 nm was measured with the Dynatech Model MR 600. Absorption spectra of tetrazolium reagents, formazan products, and cellular-generated formazans were measured with a Beckman Model MVI scanning spectrophotometer.

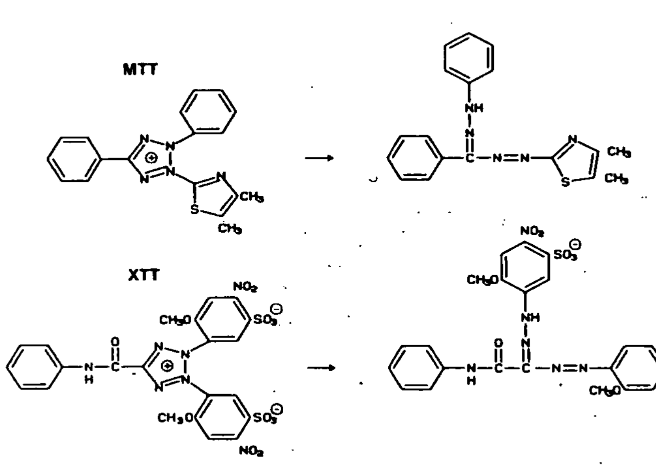

Fig. 1. Structures of MTT and XTT tetrazolium and formazan.

RESULTS

Metabolic Reduction of XTT by Human Cells. To determine the suitability of the new XTT reagent for a microcultur growth inhibition methodology, we investigated the ability of several human tumor cell lines to reduce XTT to a measurable aqueous-soluble formazan product. Table 2 shows typical MTT and XTT metabolism data for different cell inoculation densities. Two human lung tumor cell lines were incubated for different times with MTT (only 4-h data shown) or XTT, and the absorbances were measured. Cell line A549 reduced MTT far more efficiently than H322, illustrating two extremes of cellular metabolic ability. Neither cell line was able to efficiently reduce XTT during the 4-h incubation time, thus making obligatory longer incubation times for the generation of a substantial absorbance. These data also indicated that the formazan product resulting from the cellular reduction of XTT was not toxic to human tumor cells over the 96-h period of this experiment. The absorbance continued to increase, and microscopic examination of cultures confirmed that cells remained viable in the presence of the XTT-formazan. Table 1 compares absorbance measurements with MTT and XTT for several cell lines. In all cases the metabolic reduction of the tetrazolium was substantially less for XTT than for MTT (Table 1). Table 1 also

Table 2 *Metabolism of MTT or XTT*

Cell line	Cell density[a]	MTT, 4 h	XTT 4 h	XTT 8 h	XTT 24 h	XTT 48 h	XTT 96 h
		MTT/XTT absorbance[b] at the following metabolism time[c]					
A549	10	0.15	0.02	0.02	0.07	0.22	0.35
	20	0.28	0.03	0.08	0.18	0.40	0.52
	39	0.55	0.07	0.07	0.32	0.59	0.65
	78	0.95	0.14	0.14	0.44	0.85	0.97
	156	1.39	0.15	0.12	0.53	0.82	1.20
	312	1.61	0.20	0.14	0.55	0.85	1.21
	625	1.68	0.16	0.14	0.54	0.83	1.46
	1,250	1.62	0.17	0.18	0.52	0.82	1.43
	2,500	1.71	0.16	0.19	0.50	0.80	1.92
	5,000	1.62	0.18	0.27	0.52	0.82	1.85
	10,000	1.75	0.18	0.32	0.54	0.86	1.97
H322	10	0.00	0.00	0.00	0.02	0.01	0.03
	20	0.00	0.01	0.00	0.02	0.02	0.04
	39	0.00	0.01	0.01	0.02	0.04	0.06
	78	0.01	0.02	0.02	0.05	0.09	0.13
	156	0.02	0.03	0.04	0.11	0.18	0.30
	312	0.04	0.06	0.09	0.21	0.35	0.45
	625	0.25	0.09	0.15	0.34	0.41	0.63
	1,250	0.48	0.15	0.22	0.48	0.53	0.74
	2,500	0.65	0.17	0.27	0.55	0.63	0.89
	5,000	0.78	0.17	0.26	0.52	0.68	0.93
	10,000	0.86	0.15	0.23	0.48	0.65	0.91

[a] The inoculation cell density: cells inoculated/well.
[b] Data represent average absorbances minus background from triplicate wells. Culture duration was for 7 days.
[c] Plates were incubated at 37°C in 5% CO_2 for the indicated time.

illustrates the marked enhancement of the metabolic reduction of XTT in the presence of the electron-coupling agent, phenazine methosulfate (1). The addition of 0.01 or 0.025 mM PMS (0.15 or 0.38 μg/well) resulted in a marked increase in measured absorbance, and absorbance measurements generally were equal to or greater than those obtained for MTT. One complication of the addition of PMS was an increase of background absorbance (no cells in the well) with increasing concentrations of added PMS (Table 1). With the conventional MTA the liquid medium is aspirated from the assay well prior to solubilization of the formazan product. This step results in lower background absorbance with MTT in comparison to XTT, since with the soluble XTT derivative, the aspiration step is deleted. Background absorbances at PMS concentrations equal or less than 0.38 μg/well are nevertheless acceptable, since control absorbances are at least 3.5-fold greater than background at the optimal PMS concentration.

On occasion, we have observed crystal formation in microculture wells containing PMS and XTT, sometimes resulting in diminished absorbance measurements for some cell lines. The presence of such crystals in a given microplate renders the data difficult to interpret and compromises the reproducibility and validity of an experiment. In addition to high-pressure liquid chromatography and mass spectroscopy analysis of crystals, we initiated studies to examine the potential role of several experimental variables in crystal formation: pH; temperature; cell density; and incubation time. Presently we conclude that PMS is necessary for crystal formation, and that an alkaline pH (which can occur if plates are removed from their CO_2 environment for too long a time) exacerbates the problem. We do not yet have an experimental solution to this occasional interference by crystal formation; thus, until this problem is resolved, careful microscopic examination of individual microculture wells is necessary to ensure the absence of crystal formation in a given experiment. All data presented in this paper result from experiments in which crystal formation was not observed. In addition, we are examining the utility of other electron-coupling agents as a substitute for PMS in an XTT-MTA. In initial experiments, one such agent, menadione (1), has proved promising, resulting in both a manageable background and large enough absorbance values for several cell lines tested (Table 3). After careful microscopic observati n, we have yet to observe crystal formation in experiments utilizing XTT in combination with menadione; however, since our total experience with menadione is thus far more limited than with PMS, we cannot yet conclude that crystal formation is totally eliminated with this alternative electron-coupling reagent.

To evaluate the relationship between measured absorbance and viable cell number at the time of tetrazolium addition, cells were plated, allowed to attach for 1 h, and incubated with MTT or XTT plus PMS for 4 h (Fig. 2). At optimal PMS plus XTT conditions (as with MTT), absorbances peak and plateau at different inoculation densities depending on the cell line being studied. From such data (Fig. 2) a range of cell densities which give rise to a detectable and relatively linear range of absorbanc values can be determined for each cell line at a given assay duration. An extensive discussion of the effects of inoculation density and culture duration is given in Ref. 8.

XTT-metabolism data for the murine leukemia cell line, P388, are also given in Table 1. The conventional MTA requir s the use of a centrifugation step prior to medium aspiration for P388 and other suspension cell lines. Use of the XTT reagent eliminates the need for centrifugation of suspension cell lines. XTT has proved useful for other suspension cultures, including human leukemia cell lines (data not shown).

The absorbance values obtained with two human cell lines as a function of PMS concentration are given in Fig. 3. From data such as these, we have determined that all of the cell lines examined thus far yield adequately quantifiable absorbance measurements when incubated with XTT plus 0.01 to 0.025 mM PMS. Some of the cell lines which metabolized MTT less efficiently also required a larger PMS concentration to yield adequate absorbance values. The addition of PMS had little qualitative or quantitative effect on the MTT response of the cell lines tested (Fig. 3).

Spectral Characteristics of XTT Tetrazolium/Formazan. Spectral analysis of the MTT and XTT/formazan products deriv d from A549 cells in culture is shown in Fig. 4. Although the absorbance spectrum of XTT is rather different than for MTT, the addition of PMS resulted in little qualitative difference in either spectrum. The absorbance maxima for cellular-generated, DMSO-solubilized MTT-formazan and aqueous-soluble XTT/formazan are 560 and 475 nm, respectively. XTT/formazan can be easily discriminated from the background or from the unreacted XTT tetrazolium reagent.

Application of XTT to Drug Sensitivity Assays. To determine the suitability of the XTT assay for large-scale drug screening, we utilized XTT microculture methodology to generate drug sensitivity profiles for some representative standard compounds and experimental agents. Figs. 5 and 6 show drug profiles generated for Adriamycin-treated H324 cells using both the MTT and XTT reagents. The profiles were very similar for both reagents supplemented with 0.005 to 0.01 mM PMS. Lower PMS concentrations resulted in very low XTT absorbance measurements precluding accurate drug treatment analysis. The drug profiles using MTT were virtually identical for all PMS concentrations studied. Treatment of human tumor cell lines with several other experimental c mpounds resulted in comparable IC_{50} values (drug concentrations resulting in a 50% inhibition of growth) for either the MTT or XTT methodology (Table 4). Analysis of the data in Table 4 using the Spearman

Table 3 *Comparison of menadione and phenazine methosulfate in the XTT-MTA*

	MTT/XTT absorbance at the following PMS/MEN concentration[b]						
		XTT					
Cell line	MTT, 0	0.01 mM PMS	0.025 mM PMS	0.01 mM MEN	0.05 mM MEN	0.10 mM MEN	0.20 mM MEN
A549	1.947	1.433	2.110	1.065	1.107	3.057	2.973
LOX	1.026	0.403	1.514	1.059	0.789	0.740	1.573
H322	0.535	0.409	0.802	0.651	0.937	1.151	1.312
H460	2.124	1.721	2.427	0.987	1.241	2.763	3.210
MCF-7	1.443	0.553	0.737	0.459	1.434	1.299	1.551
HT-29	1.464	1.084	1.411	0.300	0.953	1.519	2.553
H23	1.042	0.423	1.241	0.217	0.472	1.121	1.324
Background ($n = 7$)	0.022 ± 0.006[c]	0.210 ± 0.010	0.191 ± 0.011	0.187 ± 0.014	0.120 ± 0.044	0.126 ± 0.038	0.109 ± 0.021

[a] Data represent average absorbance minus background from triplicate wells. Cells were inoculated at 1250 cells/well, culture duration was 7 days, and MTT or XTT metabolism was for 4 h at 37°C.

[b] PMS concentration: 0.01 mM = 0.15 μg/well; 0.025 mM = 0.38 μg/well. MEN concentration: 0.01 mM = 0.086 μg/well; 0.05 mM = 0.43 μg/well; 0.10 mM = 0.86 μg/well; 0.20 mM = 1.72 μg/well.

[c] Mean ± SD of 21 well background measurements.

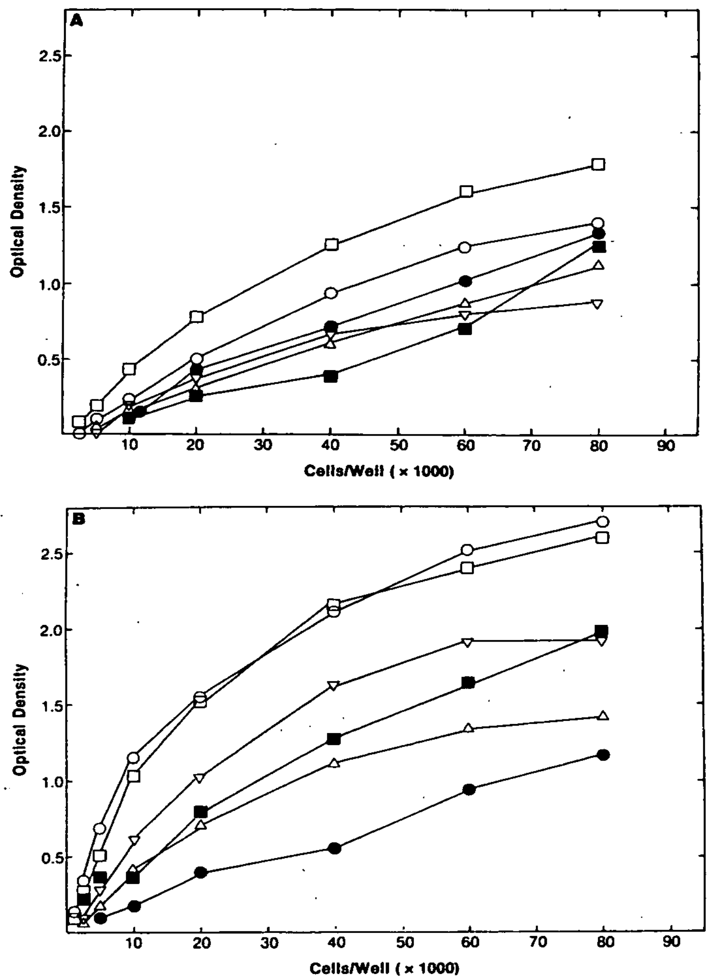

Fig. 2. Absorbance measurement as a function of viable cell density: MTT (*A*) and XTT (*B*). Viable cells were plated at the indicated cell density, allowed to attach for 1 h, and incubated with MTT or XTT plus 0.025 mM PMS (0.38 μg/well) for 4 h. A549 (○), H460 (□), MCF-7 (△), H322 (▽), LOX (●), HT-29 (■).

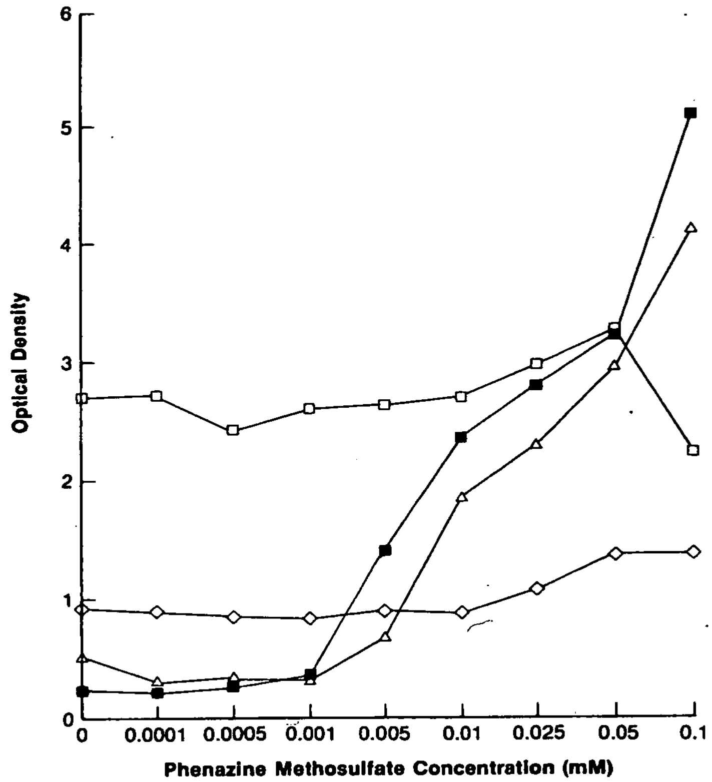

Fig. 3. Absorbance measurement as a function of PMS concentration. The PMS concentration indicated on the *abscissa* is the concentration of PMS in the XTT-PMS solution added to microculture wells. Culture duration, 7 days. A549 MTT (□), A549 XTT (■), LOX MTT (◇), LOX XTT (△).

rank-order correlation method revealed a highly significant association between IC_{50}'s derived from MTT and XTT assays ($r = 0.76$, $P = 0.0001$).

DISCUSSION

In exploring the suitability of a new microculture methodology for large-scale drug screening in the NCI/DCT drug-screening program, we initially developed a useful assay based on the reduction of MTT tetrazolium salt to a formazan product which could be easily and quickly measured in a multiwell scanning spectrophotometer system (7, 8). In this paper, we describe the evaluation of a different tetrazolium reagent which is metabolically reduced by human cells to an aqueous-soluble formazan product. Several design criteria for the new tetrazolium r agent were considered: bioreducibility of the tetrazolium; usable spectrum of the formazan product; low cellular toxicity of both the tetrazolium and formazan; and the aqueous solubility of the tetrazolium and formazan. Certain critical assay modifications were required before the new XTT reagent would approach these requirements. The XTT reagent alone proved unsuitable

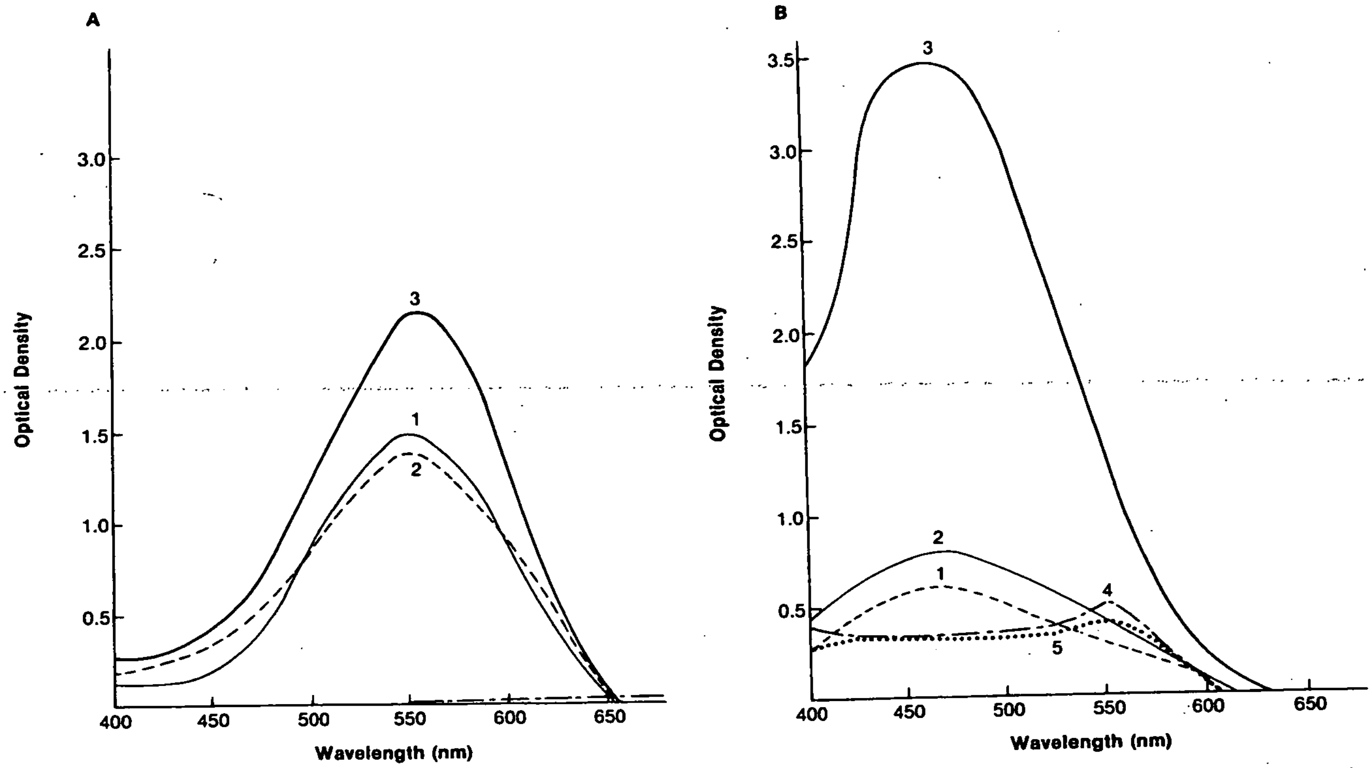

Fig. 4. Absorption spectra of MTT (*A*) and XTT (*B*) formazan products derived from cultured A549 cells (1000-cells/well inoculation, 7-day culture duration, 4-h incubation). Formazan products after incubation with the tetrazolium reagents plus: 0 mM (*1*) 0.001 mM (0.5 μg/well) (*2*), and 0.025 mM (0.38 μg/well) PMS (*3*). Nonreduced XTT tetrazolium (*4*). Background (absence of cells), tetrazolium plus 0.025 mM (0.38 μg/well) PMS (*5*).

for direct incorporation into the MTA; during a 4-h incubation, none of the human cell lines tested was able to sufficiently metabolize XTT to yield a formazan absorbance significantly greater than background. However, supplementation of the XTT incubation mixtures with the electron-coupling agent PMS resulted in adequate absorbance levels. Depending upon the metabolic capacity of a given cell line, the optimal conditions for a 4-h XTT incubation assay are 50 μg of XTT and 0.15 to 0.4 μg of PMS per well.

Fig. 2 illustrates that cellular reduction of XTT resulted in a formazan product which was not itself toxic to the cells under the assay conditions used. Cells retained the capacity to metabolize XTT for at least 96 h without evidence of toxicity. However, since metabolism times of 6 hr or less are required for our high-flux assay applications, and since the addition of XTT terminates the assay, the viability of cells after 24 h or more XTT metabolism times is not immediately relevant to the present usage. The longer incubation times resulted in both elevated absorbance measurements and increased background absorbances. XTT metabolism times of 2 to 6 h proved a suitable compromise between background and cell-generated absorbance.

The new XTT reagent used with PMS allowed application of the MTA to additional cell lines with various growth characteristics previously difficult to accommodate with the MTT-based MTA. For example, the XTT reagent greatly enhanced the usefulness of the MTA for the evaluation of cell growth and inhibition of human fibroblast cell lines (Table 2). Fibroblast cell lines are generally inefficient at metabolism of the MTT tetrazolium reagent; however, usable absorbances were obtained with XTT plus PMS. Also, the use of XTT eliminates a centrifugation step from the MTA methodology for nonadherent cell cultures. The XTT-MTA has proved usable for several suspension cell lines, including P388 and human leukemia cell lines. The XTT reagent may have an advantage for other applications of the MTA for cell growth measurements (*e.g.*, for potential antiviral compounds) where the aspiration step required by the MTT-MTA would be undesirable for either technical or safety reasons. It is beyond the scope of this paper to consider other potential uses of the XTT methodology; *e.g.*, to multicell aggregates and spheroids, however, these potential applications would appear both feasible and straightforward.

The initial experiments designed to assess the utility of the XTT-MTA in drug sensitivity assays indicate that, with certain compromises, XTT can be substituted for MTT to give comparable sensitivity and accuracy. Drug profiles obtained utilizing XTT are similar to the MTT profiles for a variety of human tumor cell lines and several experimental compounds. More extensive XTT-MTA experiments utilizing a panel of 48 human tumor cell lines treated with a wide variety of experimental drugs are under way to further explore the applicability of the XTT reagent to large-scale drug screening.[4] Results of these experiments will be detailed separately.

Even though the XTT-MTA offers several advantages over other *in vitro* assay systems, several inherent shortcomings must be considered. XTT, along with other tetrazolium approaches, depends on cellular reductive capacity, including the activity of mitochondrial dehydrogenases. The assays depend on a correlation between tetrazolium enzymatic reduction (reflected by absorbance measurements) and some associated culture characteristic such as cell number (6, 8, 9) or cell protein (8, 12). This assumption requires that cellular reductive capacity is

[4] A. Monks *et al.*, unpublished results.

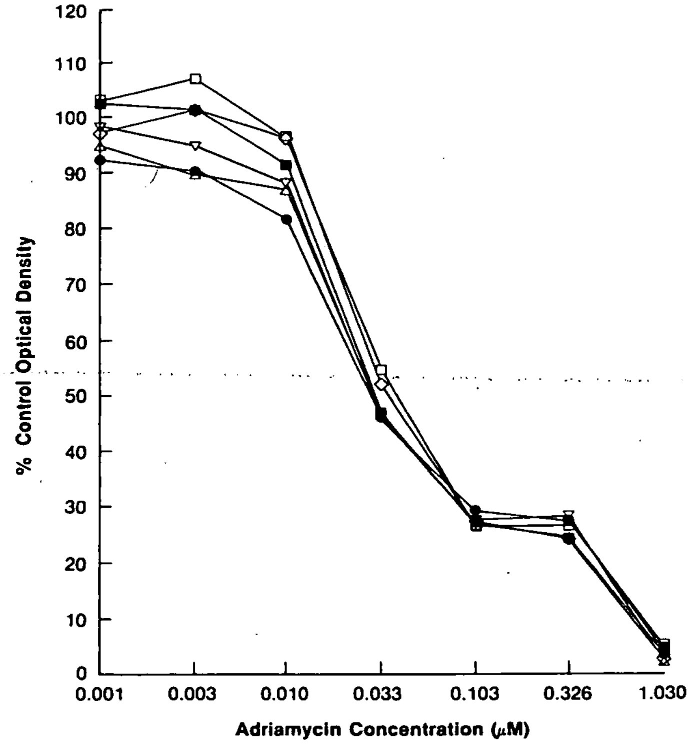

Fig. 5. Dose-response curves for Adriamycin-treated H324 cells (1000-cells/well inoculation, 7-day culture duration, 6-day Adriamycin treatment, 4-h incubation with MTT plus various PMS concentrations). *Points,* mean values from a single experiment calculated from triplicate wells subtracting background. Zero mM (□), 0.001 mM (■), 0.001 mM (◇), 0.01 mM (△), 0.025 mM (●), and 0.10 mM PMS (▽). The PMS concentrations are the concentrations of PMS in the XTT-PMS solution added to microculture wells.

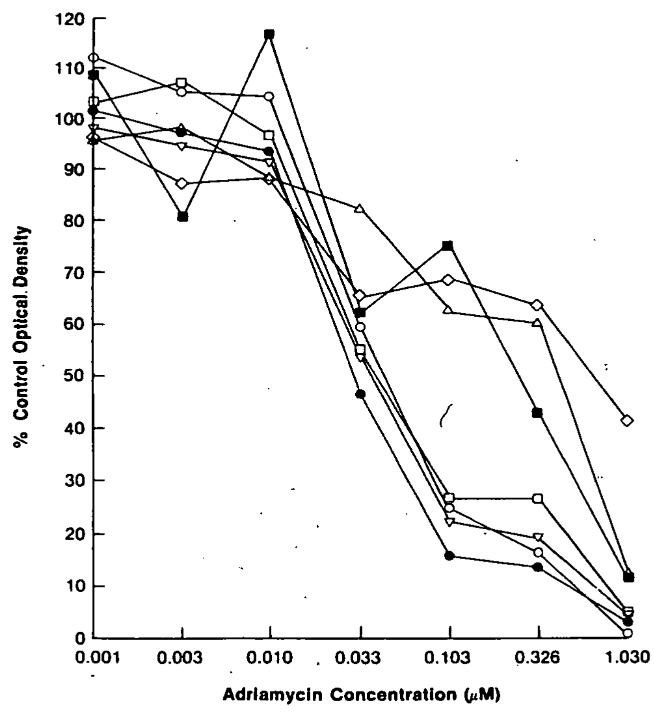

Fig. 6. Dose-response curves for Adriamycin-treated H324 cells (same experimental conditions as in Fig. 5). MTT, 0 mM PMS (□); XTT, 0 mM (■), 0.0001 mM (◇), 0.001 mM (△), 0.01 mM ( ), 0.025 mM (▽), 0.10 mM PMS (O).

Table 4 *Comparison of IC_{50}s for MTT and XTT*

Compound	Cell line	MTT	XTT
ADRI[b]	A549	1.92×10^8	2.28×10^8
	H125	2.94×10^8	6.39×10^8
	H322	5.10×10^8	4.84×10^8
	LOX	1.03×10^8	6.70×10^9
	HT-29	2.39×10^8	4.38×10^8
	MCF-7	1.97×10^8	1.77×10^8
$HgCl_2$	A549	2.15×10^5	2.16×10^5
	H125	1.35×10^5	1.93×10^5
	H322	1.54×10^5	1.52×10^5
	LOX	5.88×10^7	5.22×10^7
	HT-29	1.77×10^5	1.92×10^5
	MCF-7	1.81×10^5	1.94×10^5
BLEO	A549	5.98×10^8	8.74×10^8
	H125	2.61×10^9	1.30×10^8
	H322	1.01×10^8	1.80×10^8
	LOX	6.35×10^9	1.76×10^9
	HT-29	5.79×10^8	1.88×10^7
	MCF-7	5.56×10^7	9.31×10^7
MIT-C	A549	1.98×10^8	7.35×10^8
	H125	7.35×10^8	1.12×10^7
	H322	2.43×10^8	1.53×10^8
	LOX	3.75×10^8	1.53×10^8
	HT-29	4.17×10^8	8.19×10^8
	MCF-7	2.70×10^8	2.97×10^8
BCNU	A549	4.30×10^5	4.46×10^5
	H125	2.36×10^5	4.20×10^5
	H322	3.12×10^5	2.45×10^5
	LOX	1.30×10^5	4.76×10^6
	HT-29	4.27×10^5	7.94×10^5
	MCF-7	3.74×10^5	4.49×10^5
ACT-D	A549	2.73×10^8	2.78×10^8
	H125	2.63×10^9	3.03×10^9
	H322	2.42×10^8	1.60×10^8
	LOX	2.29×10^8	1.75×10^9
	HT-29	1.39×10^8	7.97×10^9
	MCF-7	1.43×10^8	1.36×10^8
5-FU	A549	1.45×10^6	2.02×10^6
	H125	5.36×10^7	2.73×10^7
	H322	6.24×10^7	1.67×10^6
	LOX	2.29×10^6	1.75×10^6
	HT-29	1.24×10^6	3.36×10^7
	MCF-7	7.69×10^7	7.15×10^7

[a] IC_{50} values calculated from seven concentration-dose responses. Cell inoculation densities: 1000 cells/well for all cell lines except H322 (2000 cells/well). MTA: 7-day culture duration, 6-day drug treatment, 4-h incubation with MTT or XTT plus 0.01 mM (0.15 μg/well) PMS.

[b] ADRI, Adriamycin; BLEO, bleomycin; MIT-C, mitomycin C; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; ACT-D, actinomycin D; 5-FU, 5-fluorouracil.

constitutive and remains relatively constant throughout the time duration of an experiment. However, any regulation of the cellular metabolic machinery resulting in different enzyme activity at any time will render this assumption invalid. Thus, changes of reductive capacity resulting from enzymatic regulation, pH, cellular ion concentration (*e.g.,* sodium, calcium, potassium), cell cycle variation, or other environmental factors may affect the final absorbance reading. For example, Mosman (6) has reported that mitogen-stimulated mouse spleen cells produce more MTT-formazan than do resting cells. Perturbations of these factors by experimental test compounds may further exacerbate this variability. In addition, the XTT-MTA has several unique shortcomings which must be considered before adaption of this assay for generalized drug testing. The present requirements for the addition of an electron-coupling agent increase the complexity of the cellular reduction environment (1, 13) potentially resulting in greater variability and a lack of reproducibility. PMS sometimes can cause nonspecific deposition of formazan (13), and Pearse (1) recommends that intermediate electron acceptors be avoided except to dem n-strate activities which cannot otherwise be revealed. The occasional appearance of crystal formation, as yet not completely understood, is a potential interference requiring microscopic surveillance of each individual microwell. Whereas this problem may be less significant for other applications (*e.g.,* antiviral), it

may nevertheless preclude adaption of the XTT methodology to more complex drug-screening paradigms (*e.g.*, involving large cell line panels). Substitution of a different electron transport agent for PMS in the XTT-MTA may eliminate the crystal problem; however, additional work is required to better characterize the menadione-XTT system. In addition, the relatively elevated background levels characteristic of XTT plus PMS result in the inability to utilize this method for some cell lines that exhibit poor metabolic capacity and also decrease the reliability and reproducibility of drug sensitivity measurements at drug concentrations resulting in growth inhibition greater than 80% of control values. At these levels of growth inhibition, the relatively large background absorbances generated by XTT plus PMS (or manadione) in growth medium (Tables 1 and 3) can result in signal/noise ratios of less than one.

While safety and efficiency considerations argue for the possible advantage of the XTT over the MTT-based assay for applications to high-flux drug sensitivity screens, nevertheless, there remain serious problems with the XTT-based MTA, as well as tetrazolium assays in general. Such questions should continue to be of major concern and consideration for adaption of any particular assay protocol for general usage in anticancer, antiviral, or other drug-screening programs. However, the present investigation demonstrates the feasibility of a microculture methodology utilizing a water soluble tetrazolium/formazan reagent, suggesting the inherent advantages in the development of additional reagents which might not require the use of electron-coupling agents. In addition, our present cell line panels of human tumor cell lines (8) would provide a useful resource for studying the biological activity and suitability of such new materials.

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TI The new **colorimetric assay** (WST-1) for cellular growth with normal aging and **Alzheimer**'s disease.
AU Takamatsu N
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AB A new tetrazolium compound, WST-1, a sodium salt of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, was introduced into clinical chemistry. The compound's dehydrogenase activities are useful in the colorimetric determination of the bioreducibility of cells. It proved to have a sensitivity similar to MTT, and produces a highly colored formazan dye under reductive conditions. Compared with the MTT assay, we found the WST-1 assay to be more sensitive, more convenient, and more exact. We used the new assay to study aging-related changes in human lymphocyte blastformation. Four groups of healthy adults aged 40-49 years (N = 40), 50-59 years (N = 40), 60-69 years (N = 40), 70-76 years (N = 40) were examined. Moreover, we studied lymphocytes from 16 patients (47-74 years) with probable Alzheimer's disease. For healthy controls, a negative correlation ($r = -0.3108$, $p < 0.05$) between age and enzyme activity was noted. The regression equation was: $Y = -0.0085X + 1.473$ where X and Y designate the age of the individual and enzyme activity (absorbance), respectively. On the other hand, for patients with Alzheimer's disease, age and enzyme activity did not correlate. These results indicate that the WST-1 assay may be useful in the study of changes associated with aging, and also more so than the MTT assay.
CT Check Tags: Female; Human; Male
Adult
Aged
*Aging: PH, physiology
*Alzheimer Disease: PA, pathology
Colorimetry
*Lymphocyte Transformation
Middle Age
Oxidoreductases: AN, analysis
Sensitivity and Specificity
Tetrazolium Salts
CN 0 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium); 0 (Tetrazolium Salts); EC 1. (Oxidoreductases)